Determinants of pulmonary permeability in sepsis and acute lung injury

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Summary, discussion and future perspectives

In this thesis, clinical and pre-clinical studies were bridged to demonstrate a role of the circulating angiopoietin-2 (Ang-2) protein in the pathogenesis of the pulmonary permeability of sepsis and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Plasma Ang-2 levels may have clinical value as predictors of intensive care unit (ICU) outcome, even more than the Acute Physiology and Chronic Health Evaluation Score (APACHE) II score. Furthermore, specific Ang-2 blockade may be a future therapeutic target in patients with sepsis or ALI/ARDS.

Moreover, experimental in vitro and in vivo studies from our laboratory investigating the therapeutic potential of prior statin use in the prevention of increased permeability [1] were continued in the clinical setting. The clinical study shows that prior statin therapy did not affect nor ameliorate mildly increased pulmonary permeability evoked by surgery-associated ischemia-reperfusion.

Finally, the effect of crystalloid and colloid fluid loading, a frequent therapeutic step during critical illness, on pulmonary edema was studied. The data demonstrate that clinically applied amounts of fluids per se, as long as guided by changes in cardiac output, do not negatively influence pulmonary edema or pulmonary function, independent of fluid type.

The angiopoietin-Tie2 system and pulmonary permeability

Pivotal in the pathogenesis of sepsis and ALI/ARDS is an excessive and sustained activation of the endothelium, which induces a phenotypic shift towards polymorphonuclear leukocyte (PMN) adhesion and extravasation, increased endothelial permeability and apoptosis. The angiopoietin-Tie2 system controls the responsiveness of the endothelium via multiple signal transduction pathways (Figure 1 and 2, Chapter 2).

Experimental animal studies

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are the two most studied Tie2 ligands in the context of inflammation and permeability. Several experimental studies demonstrated that Ang-1 treatment, either via administration of recombinant protein or via upregulation at genome level, protected against inflammation and vascular leakage induced by hemorrhagic

shock, Ang-2, vascular endothelial growth factor (VEGF), endotoxin or other inflammatory agents, and improved survival during endotoxemia [2-10].

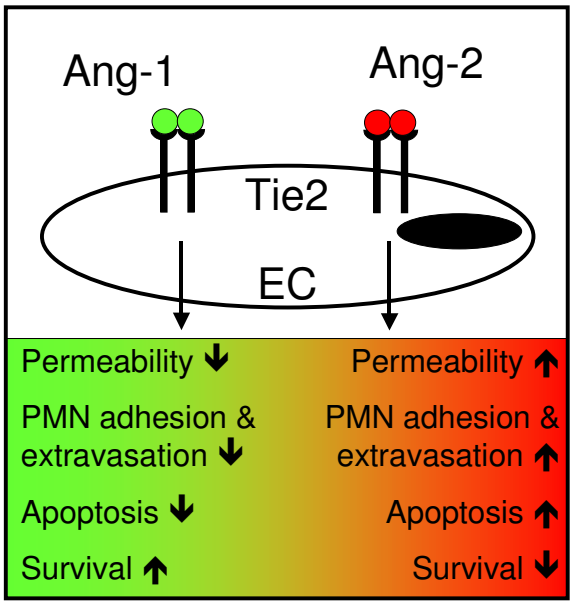

Figure 1. Simplified model of the angiopoietin-Tie2 system and the central processes in the pathogenesis of sepsis and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS)
Both Angiopoietin (Ang-1) and angiopoietin-2 (Ang-2) are ligands for the endothelial Tie2 receptor. When Ang-2 binds the Tie2 receptor, binding of Ang-1 and subsequent endothelial (EC) stabilization may be prevented so that excessive and sustained activation of the endothelium occurs. This induces a phenotypic shift towards increased endothelial permeability, polymorphonuclear leukocyte cell (PMN) adhesion and extravasation and apoptosis. Ultimately, this may result in increased pulmonary injury, pulmonary permeability edema and a reduced survival rate.

Only two studies evaluated the effect of recombinant Ang-2 on the murine lung and demonstrated that it induced pulmonary inflammation and permeability [7,11]. They did not study the hypoxic pulmonary vasoconstriction (HPV) response, another manifestation of pulmonary vascular injury. The present study (Chapter 8) showed that recombinant Ang-2 administration did not affect mice well-being or impair the HPV. Nevertheless, Ang-2, when increased during sepsis or ALI/ARDS, may enhance inflammation in the presence of other inflammatory mediators, rather than acting independently in otherwise healthy mice [12]. Ang-2 mRNA expression was increased during lipopolysaccaride (LPS)-induced lung injury in parallel with decreased phosphorylation of pulmonary Tie2

(Chapter 8). Whether the increased Ang-2 levels play a marker or a mediator during LPS-induced lung injury was subsequently studied with help of the L1-10 peptide, a specific Ang-2 inhibitor [13]. A mediator role is more likely, since hyperoxia-induced lung injury and Staphylococcus aureus-induced peritonitis were attenuated in Ang-2 knock-out mice, [11,12]. Unexpectedly, specific Ang-2 inhibition with L1-10 did not restore the LPS-induced decrease in pulmonary Tie2 phosphorylation or attenuate the LPS-induced HPV impairment (Chapter 8). This indicates that blockade of presumably circulating Ang-2 protein is not sufficient to prevent autocrine Ang-2-Tie2 interactions or prevent its role during lung injury. Nevertheless, the present data do not confirm or exclude a role of Ang-2-Tie2 interactions in the HPV. Furthermore, no conclusion can be drawn on the effect of blockade of Ang-2-Tie2 interactions on pulmonary permeability. Evaluation of the effect of LPS treatment on the HPV and on pulmonary permeability in Ang-2 knockout mice may elucidate the role of Ang-2-Tie2 interaction and downstream signaling in LPS-induced HPV impairment and increased pulmonary permeability.

Signal transduction pathways of the angiopoietin-Tie2 system: in vitro studies

In vitro studies in human pulmonary microvascular endothelial cells (HPMVECs) were performed to gain more insight in the signal transduction pathways downstream of angiopoietin-Tie2 interaction. Ang-2 interferes with constitutive Ang-1-Tie2 signaling by preventing Ang-1 from binding to the receptor, thereby acting as a natural antagonist for Ang-1. Indeed, Ang-2 completely blocked Ang-1-induced Tie2 phosphorylation (Chapter 6).

Surprisingly, Chapter 6 demonstrates that neither Ang-2, nor Ang-1 affected the basal permeability of HPMVECs, while they had opposing effects on the thrombin-induced permeability. Interestingly, angiopoietins affected permeability in the initial 15 minutes after thrombin stimulation in particular, since Ang-2 enhanced the initial permeability, while Ang-1 reduced it. In the prolonged phase of the thrombin response, angiopoietins had no effect on permeability as assessed by macromolecule passage (Chapter 6). This suggests that angiopoietins modulate the stability of the junctions during the initial rapid increase in permeability [14,15] and to a lesser extent the thrombin-induced cell contractility and gap formation. Furthermore, the data suggest that Ang-2 sensitizes the endothelium to mediator-induced pulmonary permeability and injury, rather than acting independently [12]. Indeed, this suggestion is in line with the

animal data in Chapter 8, which demonstrate that recombinant Ang-2 administration did not affect the well-being or HPV of otherwise healthy mice.

Many signal transduction molecules involved in endothelial permeability and injury are modulated by angiopoietin-Tie2 interaction (Chapter 2, Figure 2). In addition, the angiopoietin-Tie2 system also modulates the extravasation of PMNs, which accompanies the permeability response (Figure 2, Chapter 1 and 2). Endothelial permeability is regulated by a balance between tethering forces governed by vascular endothelial-cadherin (VE-cadherin) and cortical actin filament formation (F-actin) and contractile forces governed by actin-myosin interactions (Figure 2) [16]. The activation or inhibition of a certain signal transduction molecule depends amongst others on the experimental conditions, for instance basal or mediator-induced permeability and the stimulus used to induce permeability, such as thrombin, VEGF or LPS. Indeed, under basal conditions, Ang-2-Tie2 interaction increased permeability via activation of RhoA resulting in increased actin-myosin interactions [7]. In contrast, Ang-1-Tie2 interaction reduced LPS-induced permeability via increased Rac1 and reduced RhoA activity [4]. Consequently, one would expect that Ang-2 enhanced the thrombin-induced permeability via increased RhoA or reduced Rac1 activity. Nevertheless, we demonstrated rather a larger decrease than an increase in RhoA activity in Ang-2-treated cells after thrombin stimulation (Chapter 6). Therefore, Ang-2-Tie2 interaction may modulate other pathways involved in thrombin-induced permeability, such as elevation of intracellular calcium or enhancement of protein kinase C-ζ (PKC-ζ)-induced calcium influx, resulting in calcium-calmodulin and subsequent actin-myosin interactions (Figure 2). Indeed, Ang-1-Tie2 interactions had an opposing effect on those pathways [17,18]. Furthermore, the effect of Ang-2-Tie2 interaction on the distribution of VE-cadherin at the cell-cell junctions under thrombin-stimulated conditions or the tyrosine phosphorylation status of VE-cadherin, an important determinant of the stability of the junctional VE-cadherin-catenin complex [19-21] requires attention in future studies (Figure 2). In addition, Ang-2-Tie2 interactions may increase thrombin-induced permeability via enhancement of endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) synthesis, since the opposite was observed when cells were stimulated with Ang-1 [22].

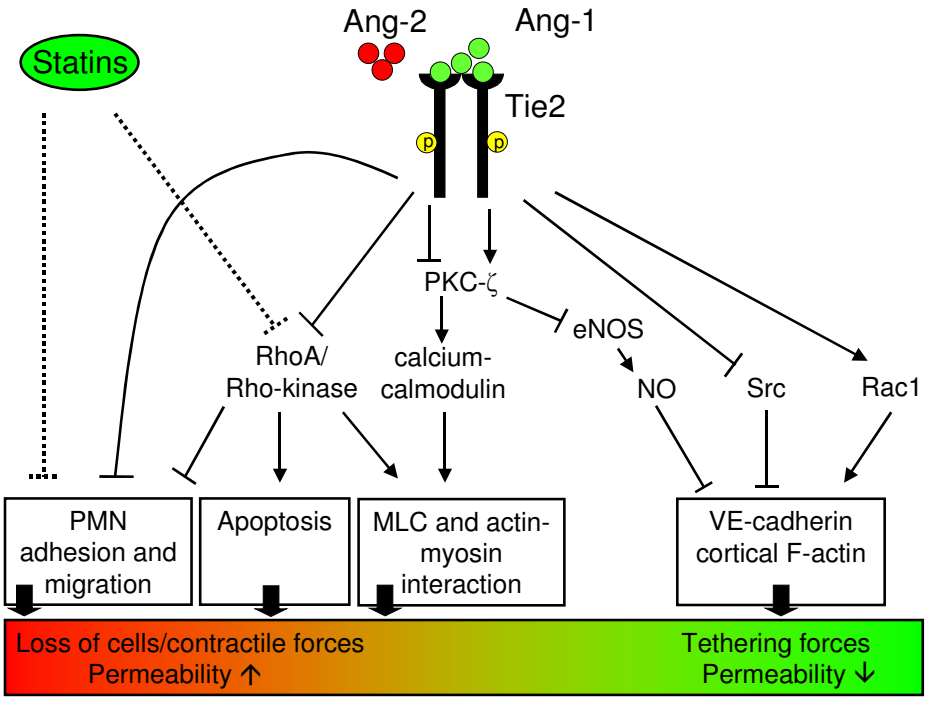

Figure 2. Angiopoietin-Tie2 interactions modulate the activity of key signal transduction molecules involved in inflammation and permeability
Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; p, phospho; PKC-ζ, protein kinase C-ζ; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; VE-cadherin, vascular endothelial cadherin; MLC, myosin light chain; F-actin, actin filaments; PMN, polymorphonuclear leukocyte.

The agonist-antagonist paradigm of Ang-1 and Ang-2 may not be absolute, since in the absence of Ang-1 or when Ang-2 is used at high concentrations, Ang-2 may also be able to activate the Tie2 receptor [23,24]. Nevertheless, in Chapter 6, Ang-2 in the absence of Ang-1 did not induce Y1100 Tie2 phosphorylation. The data do not exclude that other tyrosine residues were phosphorylated by Ang-2. Furthermore, the effect of Ang-2 on thrombin-induced permeability seemed partially independent of interference with Ang-1-induced Y1100 Tie2 phosphorylation, since cells treated with the combination had a Y1100 Tie2 phosphorylation comparable to control, but an enhanced thrombin-induced permeability (Chapter 6). Ang-2 may activate endothelial cells via another receptor, possibly another member of the receptor tyrosine kinase Tie2 family, Tie1 [25].

The angiopoietin-Tie2 system also interferes with the interaction between inflammatory PMNs and the endothelium and subsequent PMN adhesion and extravasation (Figure 2, Chapter 2) [26-28]. Furthermore, Tie2 expression on inflammatory cells [27-29] may play a role in the adhesion and migration of the inflammatory cells. Indeed, human blood monocytes, of which 20% express Tie2, performed chemotaxis towards Ang-2 in vitro [29]. Therefore, Ang-2 may recruit Tie2+ monocytes to sites of inflammation [29]. In addition, PMNs performed Tie2-dependent chemotaxis in response to Ang-1 or Ang-2 [28]. Both angiopoietins inhibited VEGF-directed migration of PMNs [28].

Detachment of apoptotic endothelial cells may contribute to increased pulmonary permeability evoked by surgery-associated ischemia-reperfusion or sepsis [16,30]. Ang-1-Tie2 interactions inhibited apoptosis via activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway [31,32] and subsequent reduction of RhoA/Rho-kinase activity (Figure 2). Indeed, Chapter 7 shows in an in vitro model that prevention of F-actin rearrangement by Rho-kinase inhibition or by treatment with an actin depolymerizator, independent of Rho-kinase, attenuated ischemia-reperfusion-induced endothelial cell apoptosis by maintaining PI3-kinase/Akt activity.

Clinical studies

In accordance with the experimental studies, Ang-2 levels related to surrogate indicators of vascular permeability and lung injury, such as low serum albumin, the lung injury score, impaired oxygenation, reduced ventilator free days and reduced compliance [7,33-35]. We were the first to demonstrate a direct positive relation between circulating Ang-2 levels and pulmonary vascular protein permeability as assessed by the pulmonary leak index method (PLI) on the first day of critical illness in septic and non-septic critically ill patients (Chapter 3). The direct relation with pulmonary permeability suggest a contributory role of Ang-2 in the pathogenesis of sepsis and ALI/ARDS. The subsequent study in septic shock patients reinforces this hypothesis by showing that plasma Ang-2 levels throughout the course of septic shock positively related to the fluid balance as a surrogate indicator of vascular permeability, pulmonary dysfunction and mortality (Chapter 4). Unfortunately, it was not feasible to measure pulmonary permeability by the PLI method in this group of patients. Nevertheless, changes in the fluid balance are likely explained, at least in part, by changes in vascular permeability, even though not reflecting pulmonary vascular permeability directly. Indeed, the fluid balance related to the

pulmonary oxygenation, suggesting that a positive fluid balance included pulmonary permeability edema.

Ang-2 levels at the first day in septic and non-septic critically ill patients and throughout the course of critical illness in septic shock patients had independent predictive value for 28 day survival (Chapter 3 and 4), in accordance with a previous study [34,35] and may even better predict mortality of critically ill patients than the APACHE II score [35,36]. Therefore, plasma Ang-2 levels of critically ill patients may be measured to predict ICU outcome. Nevertheless, the cut-off level of Ang-2 with highest sensitivity and specificity for mortality, ≥3066 pg/ml, should be validated prospectively (Chapter 4).

It is suggested that the Ang-2/Ang-1 ratio, more than absolute levels of either ligand, is critical for determining vascular permeability and injury. Indeed, the Ang-2/Ang-1 ratio, more than Ang-2 alone, showed a per-category trend from control to ARDS (Chapter 3). Nevertheless, the Ang-2/Ang-1 ratio did not relate to the fluid balance or pulmonary dysfunction and mortality throughout the course of septic shock, since Ang-1 levels were slightly, but not significantly, increased in parallel with Ang-2 (Chapter 4). Ang-1 levels in the septic shock patients were still much lower than Ang-1 levels in healthy controls [34] (Chapter 3), suggesting that the low Ang-1 levels did not offset the effects of high Ang-2 levels throughout the course. The relation between Ang-1 and pulmonary permeability and dysfunction differs between studies, since Ang-1 levels on the first day of critical illness did not relate to pulmonary dysfunction (Chapter 3), while levels throughout the course of septic shock had a positive relation (Chapter 4).

The soluble form of the angiopoietin-binding Tie2 receptor (sTie2) may modulate the mediator role of angiopoietin-Tie2 signaling in critically ill patients. Interestingly, sTie2 levels were higher in septic than in non-septic critically ill patients, in parallel with VEGF levels, and related to pulmonary permeability (Chapter 5). Nevertheless, sTie2 did not affect the negative relation between Ang-1 or the positive relation between Ang-2 and the PLI, in line with a direct mediator role of the Ang-2/Ang-1 balance or Ang-2 alone in pulmonary vascular permeability, in spite of potential binding of circulating angiopoietins by sTie2 (Chapter 5). Conversely, this suggests that sTie2 is a marker rather than a modulator of pulmonary vascular permeability in these patients.

In vitro studies showed that Ang-2 and von Willebrand factor (VWF) reside in the same secretory organelle of endothelial cells, namely the Weibel-Palade body and may be released simultaneously after activation of the endothelium [37]. Indeed, Ang-2 levels related to VWF levels, a well known marker of endothelial activation and injury associated with development and clinical outcomes of ALI/ARDS on the first day of critical illness (Chapter 3 and 4), but lost their relation on the subsequent days (Chapter 4). Although VWF may thus have an important early prognostic role, the data do not suggest a direct mediator role, in contrast to Ang-2.

Plasma VEGF correlated in some, but not in all studies, to surrogate indicators of systemic permeability [38-40]. Surprisingly, circulating Ang-2 better reflected pulmonary vascular permeability on the first day of critical illness in septic and non-septic critically ill patients than circulating VEGF, since the latter did not relate to pulmonary vascular permeability (Chapter 3). This finding is in accordance with a study in critically ill trauma patients, which showed that circulating Ang-2 related to the injury severity score, a marker of tissue hypoperfusion and a marker of activation of the complement pathway, while circulating VEGF did not [33]. Nevertheless, the present study can not exclude a relation between VEGF and permeability throughout the course of septic shock.

Prevention of pulmonary permeability edema in patients

Statins

The literature on the effect of statins on sepsis and ALI/ARDS is reviewed in Chapter 9. Up till now, the data from 31 clinical studies on statins and sepsis [41-48], ALI/ARDS [49-51] and related diseases, such as bacteremia [52-54], endotoxemia [55], multiple organ dysfunction [56], infection [57-61], pneumonia [62-68] and ischemia-reperfusion injury [69-71] have been published. Of those studies, 2 were randomized controled studies [47,55]. The clinical studies demonstrated that prior statin therapy reduced the incidence of sepsis and the mortality of patients with sepsis, ALI/ARDS or related diseases [42,46,47,52-54,56,57,59,62-67,69].

Statins may have beneficial effects beyond lowering of serum cholesterol via the so-called pleiotrophic effects, including immunomodulation, inhibition of PMN adhesion and extravasation and inhibition of endothelial permeability (Chapter

9). Experimental studies reported that statins attenuated increased permeability via prevention of RhoA/Rho-kinase activity amongst others (Figure 2). Furthermore, statins may also have beneficial effects on sepsis due to the statin-induced raise in high density lipoprotein (HDL), which can bind LPS.

Statin treatment attenuated pulmonary injury animal models [72-81] and reduced LPS-induced pulmonary inflammation in healthy subjects [55]. Surprisingly, Chapter 10 reports that regular doses of prior statin therapy, widely used in the treatment of cardiac and vascular surgery patients, did not affect nor ameliorate mildly increased pulmonary permeability evoked by surgery-associated ischemia-reperfusion in 37 patients using and 27 patients not using statins. In accordance, Kor et al. [51] reported that prior statin therapy did not affect the oxygenation ratio at the onset of ALI/ARDS. In contrast, prior statin therapy was even associated with less improvement in oxygenation ratio on the subsequent days [51]. The discrepancy between the effect observed in the experimental and the clinical studies may be explained by more severe pulmonary permeability or lung injury, the use of higher doses of statins and the continuation of statins upon lung injury development in the experimental studies [50,51]. Since statins did not have a negative effect on pulmonary permeability and a beneficial effect is strongly suggested by experimental studies, prior statin therapy should not be discontinued in the critically ill (postoperative) patients with sepsis or ALI/ARDS. When continuing statins, one should be cautious for the presumably small risk of rhabdomyolysis and aggravation of critical illness polyneuromyopathy [82,83].

In most clinical studies statins were administered to prevent cardiovascular events, not to treat infection, thus conclusions on acute statin therapy can not be drawn yet [41,42,45-49,51-54,56,57,59,60,62-67,69,70]. Interestingly, acute statin treatment improved survival in a murine model of sepsis [84] and induced a strong reduction in sepsis-related mortality in patients with subarachnoid hemorrhage [47]. Prospective randomized clinical trails on the effect of therapeutic high doses of statins on severe pulmonary permeability and outcomes of patients with sepsis or ARDS are necessary to support the beneficial effects of statins in the prevention and adjuvant treatment of sepsis or ARDS.

Fluid loading

Fluid loading is one of the most frequent supportive steps on the ICU, also in patients with increased capillary permeability in the lungs. Since a positive fluid

balance is associated with increased mortality in critically ill patients [85-87], a reasonable objective is to maintain the intravascular volume at the lowest level that is consistent with adequate circulation [88]. Indeed, a lower fluid balance is associated with a lower extravascular lung water (EVLW), reduced ventilator and ICU-days [89]. Up till recently, it was unknown what the effect of crystalloid and colloid fluids on the formation of pulmonary edema was. Chapter 11 shows that pulmonary edema and severity of lung injury are not affected by the type of fluid used for treating clinical hypovolemia in septic and non-septic patients, with more pulmonary permeability edema and higher lung injury in the former, provided that fluid loading is in the steep part of the cardiac function curve where increases in filling pressures and volumes lead to a rise in cardiac output. Indeed, in pigs with hemorraghic shock which are presumably in the steep part of the cardiac function curve, EVLW increased only 1 ml/kg when already ~ 75 mL/kg crystalloids were infused [90]. Furthermore, patients with septic shock did not develop pulmonary edema during the first 24 h of treatment, when their fluid regimen was guided by the effects on cardiac output [91]. This indicates that clinically applied amounts of crystalloids per se do not negatively influence pulmonary function [90]. The effect of loading with different fluid types beyond the plateau of the cardiac function curve, where cardiac output does not rise upon an increase in preload, is unknown.

Pulmonary capillary permeability may be even a smaller determinant of pulmonary edema formation than filtration pressures. In accordance, an experimental study reported that canine oleic acid-induced pulmonary edema was reduced by small reductions in hydrostatic pressure although they did not see effects of increasing the colloid osmotic pressure [92]. A similar beneficial effect of a lower hydrostatic pressure on mortality was observed in patients with ARDS [93].

The question remains which fluid should be given to a critically ill hypovolemic patient. Although colloids are more effective plasma volume expanders, they are more expensive than crystalloids and are associated with renal side effects and risk of anaphylaxis. Therefore, crystalloids may be preferred over colloids for fluid resuscitation.

Ratios of EVLW to blood volumes have been proposed as indirect measures of pulmonary permeability. Fluid loading may affect the relation between the EVLW ratios and the PLI. Chapter 12 demonstrates that the EVLW to blood volume ratios are determined, at least in part, by moderately increased pulmonary

permeability, relatively independent of fluid status and pressure forces in non-septic patients on mechanical ventilation with or at risk for ALI/ARDS. Normal ratios may help to exclude high pulmonary permeability.

Conclusion on potential therapeutic strategies

The studies in this thesis suggest that the pulmonary permeability of sepsis and ALI/ARDS is amongst others determined by the levels of circulating Ang-2. Furthermore, statin treatment may determine the degree of pulmonary permeability, although in the present study, prior statin treatment did not affect mildly increased pulmonary permeability evoked by surgery-associated ischemia-reperfusion. Finally, large amounts of fluids may enhance pulmonary permeability edema and pulmonary dysfunction, but clinically applied amounts of fluids per se, as long as guided by changes in cardiac output, do not negatively influence pulmonary edema or pulmonary function, independent of fluid type.

Novel causative strategies to treat increased pulmonary permeability during sepsis or ALI/ARDS may aim specifically at RhoA/Rho-kinase, since this molecule is one of the effectors of both the angiopoietin-Tie2 system and of statin treatment and is involved in increased permeability and PMN adhesion and extravasation and apoptosis (Figure 2) [94-99]. RhoA/Rho-kinase activity may be inhibited via pharmacological blockade of Ang-2 or via statin or fasudil treatment (Table 1).

Up till now, the effect of Ang-2 blockade with the blocking peptides L1-10 and L1-10 or the RNA aptamers have only been studied in animal models of angiogenesis and tumor growth, where they were successful in inhibiting those processes [13,100-102]. Nevertheless, when autocrine Ang-2-Tie2 interactions [109] play a role in the pathogenesis of pulmonary permeability, pharmacologic blockade may be a challenge, since intracellular targets are less easily reached. Furthermore, the safety profile of Ang-2 blocking in patients requires attention. An aspecific Ang-1/Ang-2 blocker AMG 386, recently used for the first time in patients with cancer, was related to the development of peripherel edema and proteinuria in some patients [110]. For patients with sepsis or ALI/ARDS, such aspecific treatment may have serious drawbacks, since not only the Ang-2, the ‘bad guy’, but also the Ang-1, the ‘good guy’, will be sequestered.

Table 1. Potential novel therapeutic targets for the pulmonary permeability of sepsis and ALI/ARDS

Target	Substance	Animal/patient; model
Ang-2	L1-10	Mice; angiogenesis [13]
Ang-2	L1-7	Mice; tumor growth, angiogenesis [100]
Ang-2	RNA aptamers	Mice, rat; tumor growth, angiogenesis [101,102]
RhoA	Statins	RCT patients; subarachnoid hemorrhage [47] Mice, rat; lung injury [72-81]
Rho-kinase	Y-27632	Mice, rat, rabbit; lung injury [103-105]
Rho-kinase	Fasudil	RCT patients; hemorrhagic stroke [106], stable angina [107] Mice; septic liver injury [108]

Ang-2, angiopoietin-2; RCT, randomized clinical trial, Y-27632 pharmacological tool for Rho-kinase inhibition in experimental studies.

Statins and the direct Rho-kinase inhibitor fasudil are already approved for the use in patients [106,107] and effectively attenuated pulmonary injury in experimental models [72-81,103-105,108]. Nevertheless, although the effect of statins on outcome of sepsis, ALI/ARDS or related diseases is currently studied in at least 12 randomized, yet unpublished, clinical trials (RCTs) (www.clinicaltrials.gov and www.controlled-trials.com) [111] pulmonary permeability or edema is not used as outcome measure. Furthermore, the effect of fasudil has only been evaluated in RCTs of hemorrhagic stroke and stable angina [106,107]. Many other direct Rho-kinase inhibitors are currently being developed [112].

Prospective randomized clinical trials on the effect of specific Ang-2 blockers, statins or fasudil in septic and ALI/ARDS patients on the pulmonary leak index for ^{67}Gallium-labeled transferrin [113,114] and EVLW by transpulmonary thermal-dye dilution [115] as measures of pulmonary permeability and edema, respectively, are required.

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